Workshop

Inducible Mouse Models

Thursday 3 July 2003

Leiden University Medical Center
Albinusdreef 2, 2333 ZA Leiden

Program

	Workshop Inducible Mouse Models, Thursday 3 July 2003	
8.30	Coffee and registration	
9.00	Opening *Sjef Verbeek*	
9.05-10.20	ER- and PR-based inducible systems *Chair: Paul Krimpenfort*	
9.05	Functional analysis of cre-ER$^{T\Delta D}$ and cre-ER^{T2} in vitro and in vivo *Ate Loonstra*	20´
9.25	New ES cell lines for the rapid generation of inducible mouse mutants *Frieder Schwenck*	20´
9.45	Villin-Cre and villin-CreER^{T2}: new tools for gene expression or deletion in the gut epithelium *Sylvie Robine*	15´
10.00	Conditional gene switching in a mammary gland reconstitution model *Jos Jonkers*	15´
10.15	Ubiquitous-inducible CrePR transgenic mice for studying conditional knockout mutants *Gloria Esposito*	15´
10.30-10.50	Coffee Break	
10.50-12.30	Advanced technologies for mouse engineering *Chair: Jos Jonkers*	
10.50	Sequenced 5´hprt and 3´hprt insertion vector libraries for chromosome engineering *David Adams*	20´
11.10	A directional strategy for monitoring Cre-mediated recombination in the mouse *Frank Schnütgen*	20´
11.30	Generation of mutant knock-in lrp mice by application of recombination mediated cassette exchange *Anton Roebroek*	15´
11.45	Monitoring Cre-mediated gene inactivation in a conditional XPA knockout model *Ingrid van der Pluijm*	15´
12.00	Double selection vectors for the construction of targeting constructs *Thierry van Reeth*	15´
12.15	A new luciferase reporter mouse that enables non-invasive imaging of Cre-mediated recombination and tumorigenesis *Paul Krimpenfort*	15´

12.30-13.30	Lunch	
13.30-14.30	Tet-regulated expression systems *Chair: Els Robanus Maandag*	
13.30	Conditional immortalization of ES cells derived progenitor cells with tetracycline controlled T-Antigen *Konstantinos Anastassiadis*	20´
13.50	Strategies for predictable quantitative regulation of gene activities via tetracyclines *Kai Schönig*	20´
14.10	Specific inhibition of gene expression using a stably integrated, inducible siRNA vector *Marc van de Wetering*	20´
14.30-14.50	Coffee break	
14.50-16.00	Applications *Chair: Marian van Roon*	
14.50	Conditional inactivation of corticotropin-releasing hormone receptor 1 *Ralf Kühn*	20´
15.10	Transgenic mice, models for Alzheimer's disease *Dick Terwel*	20´
15.30	Mice selectively deficient for interleukin-10 in T cells develop inflammatory bowel disease *Werner Müller*	15´
15.45	Physiopathological role of the mineralocorticoid receptor in heart: use of conditional transgenic models *Frederic Jaisser*	15´
16.00	Forum discussion *Chair: Hein te Riele*	60´
17.00	Drinks & snacks	

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Presentations

Functional analysis of cre-ER$^{T\Delta D}$ and cre-ER^{T2} in vitro and in vivo

Ate Loonstra

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The Cre-lox recombination system is an excellent tool for genome modification in living cells. However it is essential to know all the potential drawbacks of such a system. For example unwanted, aspecific recombinations might affect cell viability and thus influence the results. Recently others and we have shown Cre-induced chromosomal damage upon overexpression of the Cre recombinase. Inducible fusions of Cre with an estrogen receptor ligand-binding domain (CreERT) can overcome such side effects. Different CreERT fusions have been created in the past but a detailed study of recombination efficiency versus specificity has never been performed. In this study we show that one of the previously described CreERT fusions (CreER^{T2}) shows the best recombination efficiency and no toxicity under control of the ROSA26 promoter. One other fusion (CreER$^{T\Delta D}$) displays significant toxicity upon induction with 4-hydroxy-tamoxifen. In addition also presence or absence of leakiness of such an inducible system is very important. Cre activity in the absence of ligand might result in noise in your mouse model or even lead to embryonic lethality due to unwanted switching in the germ line. Also in this respect, CreER^{T2} shows a better performance than CreER$^{T\Delta D}$.
Finally, recent preliminary results suggest a possible difference between the ligand specificity of these two Cre fusion proteins. Pending confirmation of these results in the next weeks, the data will be discussed during the seminar.

New ES cell lines for the rapid generation of inducible knockout mice

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Inducible, Cre/lox mediated gene targeting is a powerful tool to analyze gene function in adult mice. Furthermore, it permits to investigate the effect of induced gene inactivation in animal disease models. This aspect is of particular interest in the validation of genes for pharmaceutical drug development, as it provides an ideal model for the action of antagonistic drugs. However, the generation of conditional mouse mutants using the current technology is time consuming as several breeding steps are required. This problem can be addressed using the recent discovery that completely ES cell derived mice can be efficiently produced by the injection of hybrid ES cells into tetraploid blastocysts.
We have generated improved fusion proteins consisting of Cre and the ligand binding domain of the human estrogen receptor. Such fusion proteins are inducible by the administration of synthetic ligands. To derive a system that allows the rapid production of inducible mouse mutants, we inserted these constructs into the Rosa26 locus through homologous recombination in hybrid ES cells. To assay the activity of the CreER fusion proteins in vitro, we introduced a Cre-LacZ reporter into the second Rosa26 allele. We demonstrate that highly efficient recombination can be achieved within 72 h of induction, whereas no background recombination can be detected in the absence of drug. We are currently generating ES cell derived mice to study the potential of our system *in vivo*.

Villin-Cre and villin-CreER^{T2}: new tools for gene expression or deletion in the gut epithelium

F. El Marjou, K-P. Janssen, D. Louvard, and S. Robine

UMR 144, Institut Curie, Paris, France in coll with D. Metzger, M. Li, P. Chambon at IGBMC, Illkirch , France.

We developed two complementary systems for Cre-mediated recombination of target genes in the mouse digestive epithelium. Firstly, Cre recombinase was expressed under control of a 9 kb regulatory region of the murine villin promoter. Deletion of loxP flanked (floxed) DNA sequences was initiated at embryonic day 9 in the visceral endoderm, and by embryonic day 12.5 in the entire intestinal epithelium. *Vil*-Cre expression and recombination were maintained in epithelia of the small and large intestine throughout adulthood. Secondly, a tamoxifen-inducible Cre recombinase (*vil*-CreER^{T2}) under control of the villin promoter was used to trigger spatio-temporally controlled somatic recombination. After induction, recombination of the reporter gene lacZ was detectable throughout the digestive epithelium. Interestingly, the recombined locus persisted for at least 60 days in the rapidly self-renewing gut epithelium after tamoxifen withdrawal. Therefore, the *vil*-CreER^{T2} line provides a unique tool to target differentiated enterocytes as well as intestinal epithelial progenitor cells.

Conditional gene switching in a mammary gland reconstitution model

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To generate mouse models of hereditary breast cancer, we have generated conditional Brca1 and Brca2 mouse mutants. Furthermore, we have generated p53 conditional knockouts to investigate possible collaboration between BRCA loss and p53 inactivation in tumorigenesis. We have crossed mice with different combinations of these conditional alleles with K14Cre trangenic mice, which express the Cre recombinase in basal cells of stratified epithelia. Using the various compound mutant strains, we were able to show that BRCA1/2 and p53 loss-of-function effectively collaborate in skin- and mammary tumorigenesis, indicating a pivotal role of the p53 pathway in BRCA1- or BRCA2-associated breast cancer.
To measure both early and late consequences of BRCA1 or BRCA2 inactivation in mammary gland epithelium, we have applied Cre/loxP-based conditional mutagenesis to a mammary gland tissue reconstitution system involving isolation, culture and transplantation of primary mouse mammary epithelial cells (MMECs). For this purpose, short-term cultures of primary MMECs from conditional mutant mice carrying a R26cre-ERT knock-in allele are subjected to tamoxifen-induced Cre expression to activate the conditional mutations prior to transplantation into cleared fat pads. Using this system we are able to effectively recapitulate BRCA2-associated tumorigenesis in a relatively short time period of 10 weeks.

Ubiquitous-inducible CrePR transgenic mice for studying conditional knockout mutants

Gloria Esposito, Marion Apotheker, Tamara Robben and Jan Gossen

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In order to analyse conditional knockout mouse models for target validation we exploited the inducible-Cre/loxP system. This system would allow us not only to overcome possible embryonic lethality but also to investigate the effect of the inactivation of a given gene at a specific stage of organ/tissue/cell development, resembling closely the effect of antagonistic drugs.
Since our conditional mutants carry loxP- flanked genes, we focused on the generation of ubiquitous-inducible Cre transgenic lines. We chose the Cre-PR system for two reasons: first, it provides a more tight control of the Cre recombinase activity when compared to other available systems and second, a number of ligands are available that e.g. are able to cross the blood-brain barrier.
We constructed conventional transgenic mouse lines carrying a cassette consisting of a Cre-PR fusion gene under the control of the CAG promoter. In an attempt to maximize the amount of Cre-PR fusion protein produced we cloned a DNA segment containing an intron and splice sites, downstream the Cre-PR gene.
In a parallel approach we inserted the same cassette in the Rosa26 locus by homologous recombination. We demonstrated the ability of our construct to generate a functional, inducible Cre-PR fusion protein *in vitro* in a transient transfection system in mouse embryonic fibroblast. A number of transgenic mouse lines has been obtained and crossed with LacZ reporter mice. The analysis of these mice is currently in progress. Initial analysis has not yet shown deletion of a floxed reporter alleles either in *ex vivo* or *in vivo* experiments.

Sequenced 5´hprt and 3´hprt insertion vector libraries for chromosome engineering

David Adams[1], Ruth Taylor[1], Tony Cox[1], Rob Davies[1], Bob Plumb[1], Louise van der Weyden[1], Jos Jonkers[2], Jane Roger[1] and Allan Bradley[1]

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Many human chromosomal disorders are caused by deletions, duplications or inversions that alter large segments of chromosome. Most of these disorders cannot be readily modelled in the mouse using standard gene targeting technologies. In 1999, we developed two insertion vectors libraries (5'HPRT and 3'HPRT) that became the basis for chromosome engineering technology and have been used extensively to generate deletions, duplications and inversions in mouse ES cells. To make these libraries more accessible to members of the mouse community we have successfully end-sequenced 62,597 clones from the 5'HPRT library and 42,056 clones from the 3'HPRT library and arrayed them against the *Ensembl* mouse genome sequence. The sequencing of these libraries has since become the basis for several other projects including the mouse genome balancer project and the development of a high throughput knockout approach. In this talk I will discuss the results of the library sequencing project, the models that have been generated using these libraries and the mouse genome balancer project.

A directional strategy for monitoring Cre-mediated recombination in the mouse

Frank Schnütgen

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Major advances have been made in using the Cre/loxP system for conditional gene ablation in mice. However, while many Cre-expressing transgenic lines are being developed, there are problems to reveal the occurrence of the Cre-mediated events at the level of individual cells. By combining the ability of Cre recombinase to invert or excise a DNA fragment, depending on the orientation of the flanking loxP sites, and the availability of both wild-type loxP and mutant lox511 sites, we devised a Cre-mediated genetic switch through which the expression of a gene is turned off, while the expression of another one is simultaneously turned on. The usefulness, efficiency and reliability of this switch to monitor, in every single cell, Cre-mediated ablation of the RARγ gene is demonstrated in the mouse. We discuss the potential of this novel approach, which will readily allow several genetic modifications in a conditional manner, including the introduction of point mutations or rescue of gene ablation. Furthermore, we will describe the idea of conditional gene trapping which is currently being realized within the German Gene Trap Consortium.

Monitoring Cre-mediated gene inactivation in a conditional XPA knockout model

Ingrid van der Pluijm

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Damage of DNA can lead to mutations and transcription/replication blocks, which can result into apoptosis and/or cancer. Cells have evolved mechanisms to repair damaged DNA. One of those mechanisms, Nucleotide Excision Repair (NER), can recognize and repair helix-distorting DNA lesions, often caused by UV. A defect in NER can lead to the disease Xeroderma Pigmentosum (XP), in which XP complementation group genes A-G are mutated. People who have XP-A are totally devoid of NER and therefore have a high risk of getting skin cancer. A mouse model for XP-A exists and it mimicks the human syndrome. Since these mice have a total NER-defect, they also have a higher DNA damage burden.

We are very interested in what happens in different tissues at different times as a result of a higher DNA damage load in relation to cancer and aging. In order to have greater flexibility in studying this, we developed a conditional XPA knockout model.

The conditional construct consists of an XPA genomic/cDNA fusion with a selectable marker, flanked by LoxP sites. By breeding these mice with Cre recombinase transgenic mice, XPA will be knocked out in an inducible, time-dependent and tissue-specific manner. The construct is designed in such a way, that after Cre excision, a LacZ-GFP fusion protein will be expressed. Cre mice we have available are ubiquitously inducible, liver inducible, skin inducible and brain constitutive.

Sofar we made the XPA conditional construct and transfected it to ES-cells. Stable clones were picked and checked for homologous recombination. Two homologously recombined clones with the right karyotype were injected into blastocysts, which resulted in a total of seven chimeras.

Two of those chimeras gave germline transmission.

At the cellular level, the LacZ-GFP fusion protein was expressed. Additionally, the XPA cDNA/genomic fusion construct was functional in both ES cells and mice. Furthermore, Cre recombinase was able to recognize and excise the flanked cDNA/genomic fusion.

Generation of mutant knock-in lrp mice by application of recombination mediated cassette exchange

Anton Roebroek

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LRP (Low Density Lipoprotein Receptor-related Protein) is a multifunctional receptor belonging to the LDL receptor family. LRP has been postulated to participate in a number of diverse physiological and pathological processes ranging from homeostasis of plasma lipoproteins, fibrinolysis and atherosclerosis to neuronal regeneration and survival. The aim of this research project is to determine the significance of many potential signals in the cytoplasmic tail of LRP in regulating the function of this multifunctional receptor. LRP will be molecularly dissected by 'Recombinase Mediated Cassette Exchange (RMCE)'. Multiple modifications (point mutations and deletions) will be introduced into the endogenous LRP gene of embryonic stem cells (ES cells) of the mouse. The impact of these modifications on the function of the endogenous LRP will be studied in vivo in subsequently generated mice.

The experimental approach started with the introduction of an exchangeable cassette in the LRP gene by homologous recombination replacing the 3'-end LRP exons to generate a parental modified ES cell line. Subsequent RMCE by transient expression of FLP recombinase can be used very efficiently (38 to 67 %) to replace the exchangeable cassette by (mutant) LRP sequences and restore the LRP gene. So far, (mutant) mice were obtained by germ line transmission from five ES cell lines: the parental ES cell line, and four ES cell lines encoding a wild-type restored LRP gene, a LRP mutant, in which the proteolytic cleavage site (furin cleavage site) was mutated and two LRP mutants, in which one of both NPXY motifs were mutated.

The RMCE methodology will be discussed in detail, together with the initial phenotypical analysis of the mutant mice.

Double selection vectors for the construction of targeting constructs

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Assembling DNA constructs for amplification in *Escherichia coli* is a prerequisite step for many experiments in molecular biology. Existing methodologies employing restriction enzymes, PCR and ligation steps are well suited for many tasks but their limitations become evident when complex engineering involving long DNA fragments are desired. One of the most tedious and time consuming type of subcloning involves the sequential addition of several nucleic acids segments (called cassettes) into a vector in order to obtain the desired recombinant. These limitations often impede the construction of targeting vectors intended for modifications of vertebrate genomes, in particular, the mouse genome via embryonic stem (ES) cells. We have developed a new system using two vectors (pTarg1 and pTarg2) this vector set use two markers (*ccdB* and *kid*) for the selection of recombinants in *E. coli*. The use of these vectors simplify the construction of targeting vectors in *E. coli*, the advantages of this approach will be discussed.

A new luciferase reporter mouse that enables non-invasive imaging of Cre-mediated recombination and tumorigenesis

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Our laboratory has made a significant investment in developing new mouse models for a variety of tumors, using Cre/Lox mediated switching of tumor suppressor genes and oncogenes. The methodology enables us to switch multiple oncogenes and/or tumor suppressor genes within cells *in vivo* at a defined time. This permits the induction of highly specific tumors within a narrow time window.
However, tumorigenesis in sporadic mouse models almost invariably exhibits a stochastic component and therefore tumor staging becomes difficult when tumors arise in or metastasize to internal organs. Sensitive in vivo imaging techniques can assist in monitoring growth and regression of tumors and allows the testing of intervention strategies at different stages of tumorigenesis.
Therefore, we have generated a transgenic mouse strain with a floxed luciferase reporter for non-invasive in vivo imaging of Cre-mediated recombination and tumorigenesis. Although luciferase imaging does not allow a high spatial resolution, it permits a rather accurate tumor mass measurement, even at small tumor sizes. We have validated the utility of our luciferase reporter strain in a lung tumor model.

Conditional immortalization of ES cells derived progenitor cells with tetracycline controlled T-Antigen

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Embryonic stem cells can differentiate into all 3 germ layers. During differentiation the totipotent ES cells will give rise to multi-, unipotent progenitor cells that will in their turn give rise to terminally differentiated cells. The process of differentiation is guided by the various factors that are added to the culture media and is not yet well understood. Our aim is to conditionally immortalize progenitor cells that arise during this process.
In order to perform this, we have modified the tetracycline regulated system so that it's application was highly predictable. The modifications were codon optimization of the reverse tet-repressor and fusion to a ligand binding domain of a steroid receptor. Furthermore the tet-controlled gene is flanked by insulator elements. These modifications abolished leakiness of the tet-repressor, insulated the tet-controlled promoter from influences of enhancer or silencer elements present in the vicinity of the integration site and showed high regulated induction upon addition of ligand and Doxycycline (Dox). Most important, these desired characteristics were observed in almost all of the clones screened.
ES cells were stable transfected with the improved reverse tet-repressor- ligand binding domain fusion and the SV40 large T-Antigen under the control of the tetracycline promoter. Expression of T-Antigen was undetectable in the absence of ligand and Dox. T-Antigen was highly induced upon addition of ligand and Dox in all the ES cell clones screened. We next tried to differentiate the ES cells into neurons and switch on T-Antigen expression at various time points during differentiation. The differentiation status of the cells was investigated by immunostaining for neuronal specific markers, at various time points, both in induced cells and in cells where T-Antigen expression was switched off.

Strategies for predictable quantitative regulation of gene activities via tetracyclines

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After insertion of a Ptet controlled transcription unit into the genome of a cell, its function will strongly depend on the characteristics of the integration site. Accordingly the vast majority of random integrations will occur in sites not optimal for tight and broad range regulation via the Tet system.
The identification of mouse lines where a Ptet controlled transcription unit is properly integrated is time consuming and costly and thus, methods to generate transgenic animals with predictable regulation properties would be of great advantage.
One strategy to achieve this goal would be the targeting of defined genomic loci, which support regulated gene expression. An alternative method could be based on large genomic DNA-fragments, which would allow to transmit the desired regulation characteristics after transgenesis-independent from the integration site.
The LC-1 mouse line contains a bi-directional transcription unit where the genes encoding CRE recombinase and luciferase are coregulated via Ptetbi-1. This mouse line exhibits superb regulation properties in all tissues examined. We have isolated the genomic locus carrying the LC-1 transcription unit as a 95kb BAC-fragment. Microinjection of the 95kb BAC-insert into the pronucleus of fertilized eggs yielded 6 founder lines, of which 5 exhibited expression patterns which closely resembled the one of the parent mouse line. The results show that the 95 kb fragment is capable of transferring with high fidelity the favorable regulation properties of the LC-1 mouse line to other individuals.
Exploiting red and Flp recombination systems, we exchanged the luc/cre expression cassette in the BAC-fragment for other inserts. We hope that the modified BAC-vectors will enable us to efficiently generate transgenic animals with predictable regulation potential either by simple DNA transfer or by direct targeting of the LC-1 locus via homologous recombination in ES cells.

Specific inhibition of gene expression using a stably integrated, inducible siRNA vector

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We have designed a doxycyclin- regulated version of the polymerase III H1 promoter, which allows inducible gene knockdown by siRNA. As proof of principle, we have targeted -catenin in colorectal cancer cells (CRC). TCF target gene expression is induced by accumulated -catenin and constitutes the primary transforming event in these cells. We have previously shown that disruption of -catenin/TCF4 activity in CRC cells by overexpression of dominant-negative TCF induces a rapid G1 arrest and differentiation. Stable integration of our inducible siRNA vector allowed the rapid production of siRNAs upon doxycyclin induction, followed by specific downregulation of -catenin. In these CRC cells, TCF reporter gene activity was inhibited, and G1 arrest and differentiation ensued. The inhibition of two more genes by this vector system demonstrates that it should be broadly applicable for inducible knock-down of gene expression.

Conditional inactivation of corticotropin-releasing hormone receptor 1

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Corticotropin-releasing hormone (CRH) plays a key role in coordinating the responses to a variety of stress-associated stimuli. Recent clinical data implicate CRH in the pathophysiology of human affective disorders. Conventional knockout of the CRH receptor 1 (Crhr1) severely impairs the stress response of the hypothalamic-pituitary-adrenocortical (HPA) system and reduces anxiety. To genetically dissect CRH/Crhr1 function modulating behaviour from those regulating endocrine function via the HPA system, we generated a conditional knockout strain in which Crhr1 function is inactivated postnatally in the forebrain. The hypothalamic and pituitary expression sites are spared such that the HPA system regulation is left intact. Conditional Crhr1 mutants display reduced anxiety while the basal activity of the HPA system is normal. In contrast to Crhr1 null mutants, conditional mutants are hypersensitive to stress: ACTH and corticosterone levels remain significantly elevated post-stress. Our data clearly show that limbic Crhr1 is modulating anxiety-related behaviour, and that this effect is independent of HPA system function.

Transgenic mice, models for Alzheimer's disease

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Our objective is to generate transgenic mice that recapitulate closely the pathology of AD. Mice are genetically modified to inactivate or overexpress genes that play a role in AD, i.e. APP, PS1, Tau, GSK-3, cdk5, ApoE, … The mice are phenotyped by a multi-disciplinary approach, i.e. biochemistry, electrophysiology, behavior, brain pathology.
Transgenic mice overexpressing APP [V717I] recapitulate robustly the amyloid pathology of AD and EOFAD: fibrillogenic Aß42 peptides are increased in brain and the mice display early behavioral defects, i.e. aggression and anxiety, learning and memory deficits with impaired LTP. Older (10-12 months) APP[V717I] tg mice invariably develop amyloid deposits in the brain parenchyma and at 15-18 months also cerebral angiopathy, as in the brain of AD patients. The APP mice are a valuable model for fundamental study of early signs and of progression of amyloid pathogenesis, and are pre-clinical test-banks for therapeutic strategies used in academic and industrial collaborations (spin-off "reMYND").
Presenilin1 is the essential catalytic component of the gamma-secretase complex responsible for intra-membranous cleavage of APP that generates the amyloid peptides. The potential of inhibition of PS1 as a therapeutic target was studied by a postnatal, neuron-specific deletion of PS1 (PS1n-/-), i.e. floxed PS1 gene x thy1- Cre recombinase. In contrast to the lethal, complete PS1 deficiency, PS1(n-/-) mice are viable and fertile and age normally. When crossed to APP[V717I] transgenic mice, the neuronal deficiency of PS1 strongly inhibited amyloid peptide formation and prevented amyloid plaque formation. That partially rescued the deficit in LTP but not the deficit in learning and memory, as measured by object recognition test. This demonstrates the noxious role of accumulating APP C-terminal fragments (C99 and others) in the cognitive deficits and advocates against gamma-secretase as a target for therapeutic intervention.
Other mice under study or construction are directed to the tau-pathology in AD, which is much less well understood than the amyloid pathology, and comprise combinations of "classic" and inducible transgenics, i.e. CaMKII- tTA with pBI-Tau-[P301L], GSK-3ß, cdk5, and p25.

Mice selectively deficient for interleukin-10 in T cells develop inflammatory bowel disease

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Interleukin-10 (IL-10) is a regulator of inflammatory responses and is secreted by a variety of different cell types. By suppressing activation of and cytokine secretion from macrophages and T helper 1 (T_H1) cells, IL-10 limits damage to host tissue by effectors of the immune system in the course of responses to microbial pathogens[1]. Herein, we address specifically the function of T cell-derived IL-10 using T cell-specific Cre/loxP-mediated gene targeting. Mice with a targeted inactivation of the IL-10 gene restricted to T cells spontaneously develop chronic intestinal inflammation and succumb to severe immunopathology upon infection with *Toxoplasma gondii*. Splenocytes from the T cell-specific IL-10 mutants activated in vitro show enhanced secretion of proinflammatory cytokines compared to cells from control animals suggesting that immune dysfunction in vivo is due to unbalanced inflammatory T cell responses. Despite unperturbed IL-10 secretion from other cell types in the mutants, their phenotype closely resembles that seen in complete IL-10 deficiency[2]. We conclude that the regulation of T cell immunity is critically dependent on T cell-derived IL-10 that cannot be substituted for by IL-10 released from other sources.

Physiopathological role of the mineralocorticoid receptor in heart : use of conditional transgenic models

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To assess the role of mineralocorticoid receptor (MR) in the heart independently of the kidney, we generated two transgenic mouse models that allow spatio-temporal control of the MR expression *in vivo*.

A first model allowed inducible expression of a 320 bp murine MR antisense mRNA (mMR-AS) was obtained in double-transgenic (DT) mice by mating donor MHC-tet OFF and acceptor tetO-mMR-AS mice. mMR-AS mRNA expression is restricted to cardiomyocytes and can be repressed when mice receive Doxycycline (Dox). DT mice progressively develop dilated hypokinetic cardiomyopathy with increased LVEDD and decreased EF and velocity shortening fraction. In DT mice, heart/body weight ratio is increased; cardiac remodeling with extensive interstitial fibrosis is observed (%, control: 0.32 ± 0.05; DT: 2.53 ± 0.31; $p<0.01$). Neither inflammation nor apoptosis are observed. Administration of spironolactone, an MR antagonist, to one mo old animals has a synergistic effect with MR antisense expression, worsening the histological and functional phenotypes. Most interestingly, the cardiopathy is reversible if transgene expression is turned off in 2-mo old animals

We are currently analysing a mirror model, i.e. with conditional, cardiac specific over-expression of the human MR. Results indicate that mice present sudden death with normal cardiac histology and no fibrosis, suggesting fatal arrythmia. Administration of spironolactone prevented premature death. Electrophysiological analyses performed on isolated cardiomyocytes revealed an increase of the length of the action potential in DT associated with an increase in whole Ca current. ECG analyses indicated an increase in PR intervals and a long QT in DT as compared to control mice. Investigations are underway to identify the underlying defects at the molecular and pharmacological levels.

Additional Abstracts

New conditional transgenic and KO mouse models as tools in the analysis of chronic inflammation

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In order to study the role of antibody effector pathways and to achieve a better dissection of the contribution of cell types and receptors involved, new animal models are being generated. In general conventional KO models provide insight in the biological function of genes however, their use is limited because deletion of a gene results sometimes in early lethality or adaptation and some genes have many functions in different cell types hampering the interpretation of the phenotype. These limitations can be circumvented by conditional KOs in which the genetic modifications are inducible and cell type specific. These models are based on the use of the Cre-loxP recombination system either combined with inducible promoters or with the fusion of the Cre recombinase to a ligand-binding domain of a hormone receptor.
As a first step we generated Fc RI-CreERT2 transgenic mice in which the expression of a Cre recombinase hormone receptor fusion protein is controlled by the macrophage (mononuclear) specific mouse FcγRI promoter whereas its functional nuclear localisation can be induced by the hormone tamoxifen.
The Fc RI-CreERT2 mouse will be crossed with mice with a 'floxed' Fc / Receptor gene. By homologous recombination two loxP sites have been introduced, within two different introns of the Fc / R gene. In the double genetically modified offspring the IgA/IgM receptor can be deleted exclusively in macrophages upon tamoxifen administration.
Moreover, the Fc RI-CreERT2 will be used to switch on apoptotic executioner genes in macrophages by Cre mediated excision of a floxed STOP sequence upstream of a recombinant gene encoding an activated, apoptosis inducing, form of the caspases.
This "lineage ablation" mouse and the conditional Fc / R KO will be instrumental for the analysis of the role of the immune system in chronic inflammation e.g. in atherosclerosis.
The design and construction of the targeting vector for the lineage ablation mouse will be discussed.

Inducible Smooth Muscle Cell Specific Disruption of P53-related Genes in Carotid Artery Lesions of ApoE Deficient Mice

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Recently, we demonstrated that adenovirus-mediated overexpression of p53 in smooth muscle cell (SMC)-rich cap of pre-existing atherosclerotic plaques of apoE deficient mice induces plaque destabilisation and rupture. We further want to investigate the role of p53-pathway in atherosclerotic plaque destabilisation and rupture *in vivo* using transgenic mouse models allowing an inducible and SMC-specific approach. To this end, we combined APOE deficient mice with mice that carry a tamoxifen-inducible Cre-recombinase under control of the smooth muscle specific SM22 promoter (SM-CreER(T2) (ki) mice, Kühbandner et al. Genesis 2000; 28:15-22), and mice that carry a conditional p53 allele (p53(loxP/loxP), Marino et al. *Genes Dev*. 2000; 14:994-1004) or a conditional allele of the p53 inhibitor mdm2 (Mdm2(loxP/loxP) mice, Grier et al. *Genesis*. 2002; 32:145-79). To assess the Cre-functionality at the level of smooth muscle cells in the atherosclerotic lesions we also combined apoE deficient mice with the SM-CreER(T2)(ki) mice and the ROSA26 reporter mouse line (Mao et al. *PNAS*. 1999; 96:5037-5042).

Application of the siRNA technology for the generation and characterization of targeted and inducible mouse models for FAP and APC-driven tumorigenesis

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Previous reports from our group have clearly shown that the generation of hypomorphic *Apc* alleles is of great relevance for the understanding of the mechanisms underlying APC-driven tumorigenesis in several tissues. We have employed the short interfering RNA (siRNA) technology towards the generation of a novel inducible mouse model where *Apc* expression levels are inducible in a tissue- and dosage-specific fashion. SiRNAs directed against short sequences (19 nt) of the mouse *Apc* gene have been developed and employed to transfect wild type ES cells in a transient fashion. A more than 10-fold activation of -catenin/Tcf-mediated transcription was achieved. These results motivated us to knock-in the most effective *Apc* siRNA 19 nt target into the 3' flanking region of the same *Apc* gene to maximize efficiency of inhibition, by homologous recombination in ES cells. Moreover, we also implemented the siRNA targeting construct with Tet-on/off elements to allow controlled induction of expression in adult animals and in a tissue-specific fashion. We are now analysing the correctly targeted ES lines for *Apc* inhibition and transduction of the Wnt signal before employing them for the generation of the novel mouse model.

Plag1, a genuine proto-oncogene, induces salivary and mammary glands tumorigenesis

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Introduction and aim: PLAG1 (Pleomorphic Adenoma Gene 1) is a zinc finger transcription factor gene that is normally expressed in a wide variety of tissues during embryogenesis but is completely repressed in adult life. In previous studies we have shown that PLAG1, is consistently rearranged in human pleomorphic adenomas of the salivary gland. This rearrangement induces an ectopic PLAG1 expression in the salivary gland. We aimed to explore PLAG 1 tumorigenesis when overexpressed in vitro and in vivo.
Material and methods: We performed in vitro experiments in which PLAG1 was overexpressed in NIH-3T3 cells. The effects were evaluated through typical markers of neoplastic transformation like focus forming assay and lost of cell-cell contact inhibition. In a first in vivo attempt, we study PLAG1 tumorigenesis by injecting cells overexpressing PLAG1 in nude mice. Both the in vitro and in vivo experiments strongly suggested that PLAG1 is a genuine oncogene. Transgenic animals are excellent models for studying the oncogenic phenotype that results from the disregulation of a known gene. We sought to mimic PLAG1 tumorigenesis by overexpressing this gene in a transgenic mouse. Preliminary results indicated a possible toxic effect of high PLAG1 level during the embryogenesis. We therefore opted for a strategy where the transgene is maintained in a silent configuration during the mouse development by using the Cre recombinase (Cre) system. The expression of Cre recombinase in MMTV-CRE transgenic mice is mainly confined to the salivary and mammary glands. Therefore, MMTV-Cre transgenic mice were selected to study the tumorigenic role of PLAG1 in both glands.
Results: In vitro experiments showed PLAG1 involvement in cellular transformation. Indeed, NIH-3T3 cells overexpressing PLAG1 lost their cell-cell contact inhibition, showed anchorage independent growth and induced tumor formation in nude mice. More conclusively results were obtained with MMTV-CRE/PLAG1 double transgenic mice that developed tumors in salivary and mammary glands. Conclusions: All together the in vitro and in vivo experiments showed that PLAG1 is a genuine proto-oncogene. Further studies in the double transgenic mice will contribute to unravel the mechanisms involved in PLAG1 tumorigenesis.

Genetic labeling of embryonic heart cells by Cre-mediated recombination

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The four-chambered heart is a structurally complex organ composed of multiple distinct components that develops from a relatively simple tube. To define the embryonic origin of the different components of the mature heart we do genetic labelling studies. Different components of the embryonic heart are labelled by Cre mediated recombination and the fate of these components is followed. We use mice that express Cre under control of the Anf promoter to label embryonic atria excluding the sinus node, the Mlc2v gene to label ventricles and atrioventricular canal, and the Mhc promoter to label myocardium but not endo- or epicardium. Furthermore, transcription factors involved in the formation of the heart are ectopically expressed in different components of the heart using Cre activated transgenes. In an attempt to make a two-component trans-activation system we have generated transgenic mice that express the 'harmless' yeast transcription factor Gal4 in the heart under control of the Mhc promoter and found that expression of this gene causes cardiomyopathy and heart failure, the severity of which correlated with the number of copies of the transgene integrated into the genome and with the expression level. Although useful for other purposes, the lines appeared to be useless for trans-activation purposes.

Generation and characterization of Tyr::Cre transgenic mice

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Organ-specific expression of a *Cre* recombinase allows the analysis of gene function in a particular tissue or cell type. Using a 6.1 kb promoter from the mouse *tyrosinase* gene, we have generated and characterized two lines of transgenic mice that express Cre recombinase in melanoblasts. Utilizing a *Cre*-responsive reporter mouse strain, genetic recombination was detected in the melanoblasts of the skin from embryonic day 11.5. In addition, Cre-expression was detected in the skin and eyes of mice. Cre transgene activity was occasionally detected in the brain and peripheral nerves but not in other tissues. When Tyr::Cre mice were crossed with mice carrying a homozygous *loxP* conditional mutation for the insulin-like growth factor receptor gene (*Igf1r*); Cre-melanoblast-specific recombination pattern was confirmed, and no abnormal phenotype was observed. In conclusion, Tyr::Cre transgenic mice provide a valuable tool to follow the cell lineage and to examine gene function in melanocyte development and transformation.

Generation of Alpha-fetoprotein knockout mice

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Alpha-fetoprotein (AFP) is the major embryonic serum protein. Its synthesis is swichted off at birth but can be restarted in case of liver regeneration or hepatomas. Our laboratory created an KO mice for the Afp gene by replacing the first two exons of the gene by a lacZ-lox-neo-lox cassette. We show that mice homozygous for the Afp null allele develop normally. Single blastocyst implantation experiments demonstrated that fetal AFP is not essential for embryonic development and viability.
Adult females are sterile due to an anovulation. Ovaries contain follicles at different stages of differentiation, including the pre-ovulatory Graafian stage. Experiments of ovarian transfert and superovulation showed that the ovaries of those mice are functional but lack signal coming from the hypothalamus-pituitary system. LH/FSH balance is abnormal. AFP, probably via its capacity to bind estrogen, plays thus an important role in the proper development of the female hypothalamus-pituitary system.

Endothelial specific inducible Flk1Cre and Tie2Cre mice for cell tracing studies

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Neointimal cells are described to be derived from medial smooth muscle cells, fibroblasts, and marcophages. Recent data indicate that also endothelial cells can transdifferentiate into mesenchymal intimal cells. Inducible Flk1Cre and Tie2Cre mice (endothelial specific) and SM22Cre mice (smooth muscle specific) crossed with Rosa26floxed lacZ line appeared to be a powerful recombination system for cell tracing studies in mammals. Using a truncated Wnt promoter we are able to study specifically the role and fate of the neural crest-derived smooth muscle cells during vessel wall formation and maturation, and during pathological circumstances (TGFbeta2 ko).

Targeted integration of the modified Tet repressor-VP16 activator

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We are constructingan E14 ES line with a targeted integration of the modified Tet repressor-VP16 activator. This will allow constitutive and reproducible expression of the gene, both in the ES cells and in derived transgenic mice.

Generation of bone and cartilage specific APC knock out mice

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The recent identification of inactivating and gain-of-function mutations in LRP5, a wnt co-receptor, in two distinct bone and mineral disorders have indicated a fundamental role for wnt-singalling in skeletal formation. The APC-gene plays a central role by integrating wnt-signals from the cell membrane to the nucleus. It is presently unclear whether APC plays a role in the development of the bone fenotype in patients with distinct mutations in LRP5. The aim of the project is to generate osteoblast and chondrocyte specific APC knock-outs to study the role of the APC-gene and hypomorphic APC-variants in bone formation in more detail. We have obtained mice expressing the CRE-enzyme under the control of the osteoblast specific 2.3kb fragment of the mouse col1 promoter and under the control of the chondrocyte specific rat col2 promoter, respectively. Presently we are crossing these lines with various transgenic APC mutant mice.

Kidney epithelial cell-specific expression of a ligand-inducible Cre recombinase

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We are developing mice expressing a Cre-recombinase that facilitates gene targeting exclusively in kidney epithelial cells, and in a temporal controlled manner. We are using 2 inducible systems: 1. the CreER^{T2}–system, in which Cre becomes activated upon tamoxifen treatment, and 2. the Tet On system (M2 variant), in which Cre recombinase becomes expressed upon doxycycline administration.
Transient transfections with our Cre-ER^{T2} construct showed that Cre recombinase was expressed in cultured renal epithelial cells, as demonstrated by immunostaining. In the absence of tamoxifen Cre was detected in the cytoplasm, but when tamoxifen was added to the medium, Cre was almost exclusively observed in the cell nucleus. Transient transfections with the doxycycline-inducible construct showed that Cre recombinase is hardly or not detectable in the absence of doxycline and highly expressed in cultured renal cells in the presence of doxycycline. Transgenic mice will be generated with these constructs and characterized.

Using the GeneSwitch system to study the roles of Wnt growth factors and their antagonists during development

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To understand the What, When and Where of Wnt signaling during mouse development, we will focus on signaling occurring in the extracellular space, as it is the primary source of information used by embryonic cells to define their fate and ultimately the formation of the embryo. First, a gain-of-function study will be conducted by generating transgenic mouse lines containing inducible Wnt transgenes. Second, "loss-of-function" experiments of Wnt signaling will be carried out by controlled overexpression of secreted dominant-negative mutant receptors or secreted Wnt antagonists.

We plan to use the GeneSwitch system to trigger the overexpression of Wnts or Wnt antagonists at specific time points during the process of mesoderm induction and patterning. We have already generated several lines containing the regulatory construct flanked by insulator sequences and are in the process of making transgenic mice with the inducible constructs.

The phenotypes will then be studied in embryos after induction (using mifepristone) of the Wnts or dominant-negative receptors to answer the questions: Which operation requires the specific Wnt signal under scrutiny, when and to what extent?

Development of vectors that combine the tet-system and the Flp-system

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Tet-dependent expression systems are widely used for regulated gene expression. However, the regulation capacity is strongly dependent on the site of integration. This makes the screening for well-regulated cells or animals laborious and time consuming. To simplify the generation of highly inducible clones we developed vectors, which combine the tet-system and the Flp-system. In a first step the cells are tagged with an autoregulatory tet vector. This vector allows an easy screening based on the expression of reporter genes like luciferase or GFP. In a second step the screening cassette is exchanged for another cassette harbouring the gene of interest using the Flp recombinase. In the past we developed this system for established cell lines and expand it now to ES-cells as well as to primary cells.

Novel conditional expression constructs for site specific knock-in at the ROSA26 locus

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A series of three conditional expression constructs for site specific knock-in at the ROSA26 locus were generated as a platform for screening the function of novel secreted proteins *in vivo*. Expression of the secreted factor is Cre activity dependent, allowing the imposition tissue specific and/or temporal regulation. Expression may be monitored by GFP activity as the Hyg-EGFP fusion is conditionally expressed as a bi-cistronic message. The three expression constructs differ in their use of promoter; either endogenous ROSA26 promoter, CAG promoter or EF1-alpha promoter. The ROSA26 and CAG promoter versions have been tested in murine ES cells for their ability to support Cre activity dependent expression.

Generation and characterization of a novel inducible mouse model for APC-driven tumorigenesis: *Apc*^{15Lox}

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Both in man and mouse, the hereditary mutation in the *APC* (adenomatous polyposis coli) tumor suppressor gene leads to intestinal tumors due to functional loss of the wild-type *APC* allele. Previous reports from our group and others have underlined the importance of the role played by *Apc* in development, differentiation and tissue homeostasis. To conditionally inactivate the *Apc* gene in the mouse, we have generated a new conditional *Apc* allele, *Apc*^{15lox}, where exon 15 is flanked by two *loxP* repeats. Cre-recombinase expression leads to a truncated mutant allele, *Apc*$^{\Delta 15}$, similar to those observed among very severe FAP (familial adenomatous polyposis) patients. *Apc*$^{+/15lox}$ mice were bred with the EIIa*Cre* deleter mouse. EIIa*Cre*;*Apc*$^{+/15lox}$ mice develop a high number of intestinal tumors indicating that the Cre-induced deletion occurs at high efficiency. *Apc*$^{15lox/15lox}$ mice are now being crossed with transgenic mice where Cre is controlled by tissue-specific promoters such as those of the *Mck* gene (striated muscle and heart), *Fabp* gene (intestine), etc. to evaluate the effects of loss of Apc function in specific organs.

Conditional mutagenesis to study mechanisms underlying immune homeostasis

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We apply conditional mutagenesis to study mechanisms underlying immune homeostasis. Our current programme focuses on the role of the TGF-β system, which is known to control the differentiation and activity of most cell types through modulation of the transcriptome. To identify the regulatory pathways targeted, we generated mice lacking the TGF-β receptor in B cells and identified, making use of comparative microarray analysis, the pathways targeted by TGF -β to control B cell responses (Roes et al PNAS 2000). We are pursuing similar studies in other leukocyte subsets including granulocytes and macrophages to obtain a comprehensive picture of the TGF-β induced mechanisms in functionally distinct cell types. We thus hope to distinguish cell type specific from more general homeostatic pathways and to identify novel genes and pathways involved in the control of leukocyte activity and immune homeostasis. By generating an integrated data set on TGF-β modulated genes we may be able to identify, by bioinformatics, patterns in regulatory DNA that enable the coherent cell type specific effects of TGF-β receptor signalling.

Cre mice for a knockout in specific cells of the inner ear

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We have been trying to produce and/or obtain from other labs several types of cre mice to do a knock-out in specific cells of the inner ear. We have been using two unpublished mouse lines obtained from different labs in which cre expression is regulated by hair-cell-specific promoters, without positive results (cre already active in the egg resulting in lethality). I've tried to make a rhombomere 4-specific cre mouse line without any result (9/9 lines showed virtually no cre-expression in rhombomere 4). Furthermore we are breeding our mice with a keratin K14-specific cre mouse line (Jonkers et al., Nat. Genet. 29, 418-425, 2001) to investigate epidermis and hair follicles, and possibly also in mammary gland epithelium.
Finally, I'm interested in an inducible, ubiquitously expressed cre mouse line to turn off expression of target genes throughout the body at specific stages of embryonic development or later.

The development of an inducible CD70 expressing transgenic mice

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One of the receptor-ligand pairs involved in the regulation of the immune response is CD27-CD70, it belongs to the TNF-R/ TNF family and acts as a costimulatoir signal early after antigenic stimulation. Extensive studies (Ramon Arens, Immunity) showed that overexpression of the CD70 gene in B cells in a transgenic mouse model led to an enhanced level of effector cells, accompanied by increased levels of IFNy.
However, presumably due to the high levels of IFNy, the B cell pool is being depleted within 8 to 12 weeks. At later age the mice even develop more immune deficiency, since also the T cell pool is reduced by means of chronic activation. Eventually the mice will die at relatively young age, caused by opportunistic infections. Therefore we want to develop an inducible CD70 expressing mice, to study the role of temporarily high expression of CD70, leading to enhanced CD27 signaling. Our hypothesis will be that the mice will benefit from this enhanced signaling, since the development of effector cells will be increased. We will use the tet on / tet off system to ensure that the transgene will not be expressed under normal conditions, but only after the addition of tetracycline. Currently we are cloning the construct which will generate the tissue-specific expresion of both the enhancer and the repressor of the tet operator sequence.

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